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Contribution of NADH oxidase to oxidative stress tolerance and virulence of *Streptococcus suis* serotype 2

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Abstract

Streptococcus suis is a major swine and zoonotic pathogen that causes severe infections. Previously, we identified two Spx regulators in *S. suis*, and demonstrated that SpxA1 affects oxidative stress tolerance and virulence. However, the mechanism behind SpxA1 function remains unclear. In this study, we targeted four genes that were expressed at significantly reduced levels in the *spxA1* mutant, to determine their specific roles in adaptation to oxidative stress and virulence potential. The Δ*nox* strain exhibited impaired growth under oxidative stress conditions, suggesting that NADH oxidase is involved in oxidative stress tolerance. Using murine and pig infection models, we demonstrate for the first time that NADH oxidase is required for virulence in *S. suis* 2. Furthermore, the enzymatic activity of NADH oxidase has a key role in oxidative stress

tolerance and a secondary role in virulence. Collectively, our findings reveal that NADH oxidase plays an important part in SpxA1 function and provide a new insight into the pathogenesis of *S. suis* 2.

Key words

Streptococcus suis; NADH oxidase; oxidative stress; virulence

Introduction

Streptococcus suis is a major swine and zoonotic pathogen responsible for severe economic losses in the swine industry and an increasing number of human cases.[1] It causes a wide range of diseases in pigs, including meningitis, septicemia and endocarditis.[2] *S. suis* infections in humans lead to meningitis and streptococcal toxic shock-like syndrome (STSLS).[2] In total, 33 serotypes (types 1 to 31, 33, and 1/2) of *S. suis* have been proposed based on capsular polysaccharides,[3] of which serotype 2 is the most common cause of infections in humans and pigs worldwide.[4] The first human case of *S. suis* infection was reported in Denmark in 1968.[4] By 2012, the total number of *S. suis* infections in humans was close to 1600 cases, doubling the number published in 2009.[5] Remarkably, two large outbreaks of *S. suis* epidemics occurred in China in 1998 and 2005, which resulted in 25 human cases with 14 deaths and 215 human cases with 38 deaths, respectively.[6] In addition, *S. suis* has been reported to be the major cause of adult meningitis in Vietnam, the second in Thailand and the third most common cause of community-acquired bacterial meningitis in Hong Kong.[7] Although numerous studies have been performed over the past 40 years, the pathogenesis of *S. suis* infection is still not entirely known.

During the infection process, bacteria encounter changing environments and host factors. Transcriptional regulators play an important role in response to environmental signals by modulating the expression of related genes. Spx proteins are a group of global regulators found in low-GC content Gram-positive bacteria, and have been shown to be involved in stress responses and virulence.[8-10] In previous work, we identified two orthologs of the Spx regulator in *S. suis*, namely SpxA1 and SpxA2, and demonstrated that SpxA1 affects oxidative stress tolerance and virulence.[11] Microarray analysis revealed that several genes possibly involved in oxidative stress responses and/or virulence were significantly downregulated in Δ*spxA1* compared to the parent strain.[11] These included *nox* (SSUSC84_0648, encoding NADH oxidase), *tpx* (SSUSC84_1246, encoding thiol peroxidase), *copA* (SSUSC84_1247, encoding copper-transporting ATPase), and *sodA* (SSUSC84_1386, encoding superoxide dismutase).[11] Genes *nox*, *tpx* and *sodA* have been well studied and

reported to be involved in oxidative stress responses and/or virulence in various streptococci and other bacteria,[12-29] while *copA* has been shown to be implicated in copper resistance.[30-32] Additionally, an undefined gene, *0350* (SSUSC84_0350, encoding a hypothetical protein) is potentially involved in oxidative stress and/or virulence, as it was downregulated 8.7-fold in Δ*spxA1*.[11] Functional analysis of these SpxA1-regulated genes will be undoubtedly important for the understanding of SpxA1 function and gaining insights into the pathogenesis of *S. suis* infection. However, of these genes, only *sodA* has been described in *S. suis*.[26, 28] The other four genes have not been characterized yet, and their biological function in *S. suis* remains unclear.

In this work, we examined the roles of the genes *0350*, *nox*, *tpx* and *copA* in oxidative stress tolerance and virulence of *S. suis* 2. The Δ*nox* strain exhibited increased susceptibility to oxidative stress agents and attenuated virulence in murine and pig infection models. Furthermore, the NADH oxidase activity has a key role in oxidative stress tolerance and a secondary role in virulence.

Results

Roles of four genes in oxidative stress tolerance and virulence of *S. suis* 2

To investigate the roles of the genes *0350*, *nox*, *tpx* and *copA* in oxidative stress tolerance, the ability of the mutants (Δ*0350*, Δ*nox*, Δ*tpx* and Δ*copA*) to grow under low- and high-oxygen conditions was examined and compared with the wild-type (WT) strain. Under static conditions (i.e. low oxygen tension), growth of Δ*nox* was slightly reduced compared with the WT strain, while growth of the other three mutants was essentially identical to that of the WT strain (Figure 1A). In contrast, growth of Δ*nox* was severely impaired under vigorous shaking (i.e. high oxygen tension) (Figure 1B). Δ*tpx* also showed an obvious growth defect under these conditions, while Δ*0350* and Δ*copA* showed no major difference in growth compared to the WT strain (Figure 1B). We also evaluated the ability of the WT and mutant strains to grow in the presence of H_2O_2, and found that Δ*nox* grew poorly compared to the other strains (Figure 1C). These results suggested that *nox* is involved in resistance to oxidative stress generated by environmental oxygen and hydrogen peroxide.

The roles of the four genes in *S. suis* virulence were examined using a murine infection model. Mice infected with the Δ*nox* mutant showed no clinical signs and all survived (Figure 1D). However, mice in other groups developed typical clinical symptoms of *S. suis* 2 infection, including rough hair coat, lethargy, and swollen eyes. The final survival rates of mice in the Δ*0350*, Δ*tpx* and Δ*copA* groups were 37.5%, 12.5% and 25%, respectively, compared to 12.5% in the WT group (Figure 1D). The survival rates were significantly lower in the WT-infected mice than in the Δ*nox*-infected mice ($P = 0.0004$, the log-rank test). The data indicated that *nox* is required for *S. suis* infection.

Taken together, these results revealed that *nox* plays a key role in oxidative stress tolerance and virulence of *S. suis* 2. Therefore, further research focused on the *nox* gene, and the complementation strain CΔ*nox* was included in all experiments.

Bioinformatics analysis of *S. suis* NADH oxidase

In the genome of *S. suis* 2 strain SC84, the *nox* gene is annotated to encode NADH oxidase. BlastN analysis using the *nox* sequence of strain SC84 confirmed the presence of *nox* in all 23 complete *S. suis* genomes available in the National Centre for Biotechnology Information database as of 31 March 2016 (Supplementary Table 1). Multiple sequence alignments of NADH oxidase from *S. suis* and other streptococci revealed that NADH oxidase is highly conserved among streptococcus species (Supplementary Figure 1A). Protein homology modeling was performed to predict the structure of *S. suis* NADH oxidase (Supplementary Figure 1B), which may be useful for studying its active sites and the design of therapeutics. The secondary structure of *S. suis* NADH oxidase is predicted to consist of 11 α-helices, 25 β-sheets, and 5 coils (Supplementary Figure 1A).

The Δ*nox* strain exhibits reduced tolerance to oxidative stress agents

In the preliminary study, we showed that *nox* is involved in oxidative stress tolerance. To avoid any possible polar effect caused by *nox* deletion, the same experiments were carried out with the WT, Δ*nox* and CΔ*nox*

strains. As expected, Δ*nox* exhibited impaired growth under vigorous shaking or in the presence of H_2O_2, while CΔ*nox* grew as well as the WT strain under all conditions (Figure 2A-C). Next, we examined the sensitivity of *S. suis* strains to SIN-1, which indirectly generates $ONOO^-$.[33] In the presence of 2 mM SIN-1, the growth of Δ*nox* was markedly delayed (Figure 2D). We also evaluated the ability of these strains to grow in the presence of paraquat, which generates intracellular $O_2^{\cdot-}$ radicals. However, no obvious difference in growth was observed between Δ*nox* and the WT strain (Supplementary Figure 2). These results strongly suggested that *S. suis* NADH oxidase plays a role in oxidative stress tolerance.

NADH oxidase contributes to the virulence of *S. suis* in the murine infection model

To confirm that the lack of *nox* was responsible for the impaired virulence of Δ*nox*, groups of ten mice were inoculated intraperitoneally with ~1.5×10^8 CFU of the WT, Δ*nox* and *C*Δ*nox* strains to determine survival rates. As expected, mice in the Δ*nox* group exhibited no clinical signs and the survival rate was 100%, while mice in the WT and CΔ*nox* groups showed severe clinical symptoms, and the survival rates were 20% and 0, respectively (Supplementary Figure 3). The survival rates were significantly higher in mice infected with the Δ*nox* mutant than in those infected with the WT strain ($P = 0.0003$, the log-rank test), and the CΔ*nox* strain ($P < 0.0001$, the log-rank test). Therefore, the reduced virulence of Δ*nox* was due to the deletion of *nox*, not a possible polar effect.

To further investigate the nature of the reduced virulence, bacterial counts of these strains in the blood and organs (brain, liver and spleen) of mice infected with a sublethal dose of bacteria were determined at 6 and 12 h after infection. Bacterial counts in the blood (Figure 3A), brain (Figure 3B), liver (Figure 3C) and spleen (Figure 3D) of mice in the WT and CΔ*nox* groups reached high levels at 6 h after infection, then decreased to relatively lower levels at 12 h after infection. However, no bacterial cells could be detected at 6 and 12 h after infection in the blood and organs of mice infected with Δ*nox* (Figure 3). These results indicated that *S. suis* NADH oxidase is involved in colonization of the blood and organs during animal infection.

Considering that inflammation plays an important role in *S. suis* infection,[34] we compared the capacity of the strains to induce inflammatory mediators. The production of both TNF-α and MCP-1 was severely reduced in mice infected with the Δ*nox* mutant in comparison to animals infected with the WT and CΔ*nox* strains (Figure 4). Mice in the WT group reached peak production of TNF-α (Figure 4A) at 6 h and MCP-1 (Figure 4B) at 9 h after infection. Serum levels of TNF-α (Figure 4A) and MCP-1 (Figure 4B) from CΔ*nox*-infected mice were similar to those from P1/7-infected mice. In contrast, the Δ*nox* mutant triggered a very low production of both TNF-α (Figure 4A) and MCP-1 (Figure 4B).

NADH oxidase facilitates the growth of *S. suis* in blood

To test whether *S. suis* NADH oxidase plays a role in evasion of innate immune responses, we examined the ability of the WT, Δ*nox* and CΔ*nox* strains to grow in whole blood collected from BALB/c mice. The mean growth factors of Δ*nox* after 1-, 2-, and 3-h of incubation were 0.912, 1.088 and 1.797, respectively, while those of the WT strain were 2.448, 9.387 and 42.053, respectively (Figure 5). Furthermore, the growth factors of the CΔ*nox* strain were restored relative to the mutant, though they did not reach the level of the WT strain (Figure 5). Growth of the Δ*nox* mutant in whole blood was significantly slower than that of the WT and CΔ*nox* strains ($P < 0.0001$, two-tailed unpaired *t* test), suggesting that *S. suis* NADH oxidase has an effect on immune evasion.

The Δ*nox* mutant is attenuated in the pig model of infection

To confirm the observed impaired virulence of the Δ*nox* mutant, we conducted a trial in pigs, which are the natural hosts of infection. Animals in the control group did not present any clinical signs during the test. Conversely, all pigs infected with the WT strain developed most of the typical symptoms, including depression, prostration, swollen joints and shaking within 24 h. Two of them died or were sacrificed for ethical reasons at day 3 post-infection and two others at day 4 post-infection. In contrast, animals in the Δ*nox* group showed no signs of infection and all survived until the end of the trial. In the CΔ*nox* group, three of five pigs presented severe clinical signs and died within 3 to 4 days, while another two showed mild

symptoms and survived. Significant differences in survival rates were found between the Δ*nox* group and the WT group ($P = 0.0144$, the log-rank test), and between the Δ*nox* group and the CΔ*nox* group ($P = 0.0486$, the log-rank test) (Figure 6A).

Histopathological studies were carried out to examine the pathological changes in brain, heart and lungs of the infected pigs. The meninges of pigs in the WT group were severely thickened and a mass of inflammatory cells could be observed (Figure 6B). Similar pathological alterations occurred in the meninges of CΔ*nox*-infected pigs, while the meninges of Δ*nox*-infected pigs showed no obvious changes (Figure 6B). In the heart of pigs inoculated with the WT strain, parts of myocardial fibers arranged disorderly, accompanied by myocardial cells edema and degeneration (Figure 6C). By contrast, there were no obvious changes in the heart of Δ*nox*-infected pigs (Figure 6C). Additionally, disordered arrangement of myocardial fibers and disappearance of transverse striations were observed in the heart of CΔ*nox*-infected pigs (Figure 6C). Pathological characteristics in the lungs of pigs in the WT and CΔ*nox* groups were also quite distinct from those of pigs infected with the Δ*nox* mutant. Pigs challenged with the WT and CΔ*nox* strains exhibited thickened alveolar walls, expansive capillaries, serous effusion and infiltration of inflammatory cells (Figure 6D). However, animals in the Δ*nox* group displayed no obvious pathological changes (Figure 6D).

Taken together, the experimental infection on pigs also indicated that NADH oxidase contributes significantly to the virulence of *S. suis* 2.

The NADH oxidase activity plays a key role in oxidative stress tolerance and a secondary role in virulence

To detect the enzymatic activity of NADH oxidase, we prepared recombinant NADH oxidase (rNox) by cloning the *nox* gene into pET-30a vector and introducing the resultant plasmid into *E. coli* BL21 (DE3) strain for expression. As seen in Figure 7A, purified rNox corresponded to an active form with a high activity (6258.2 U/mg) compared with NADH oxidase expressed in engineered *Saccharomyces cerevisiae* strains.[35] We also generated two inactive forms of NADH oxidase with point mutations of the putative active sites

(H11A and C44A). Both inactive forms displayed significantly reduced activity compared to the WT NADH oxidase (Figure 7A).

In addition, we constructed two complementation strains of Δ*nox* with the inactive NADH oxidase, i.e. CΔ*nox*(H11A) and CΔ*nox*(C44A). These two strains exhibited severely impaired growth under vigorous shaking or in the presence of H_2O_2, while their growth was almost identical to that of the WT and CΔ*nox* strains under static conditions (Figure 7B-D). Moreover, the survival rates were lower in mice infected with CΔ*nox* than in those infected with CΔ*nox*(H11A) ($P = 0.1624$, the log-rank test) and CΔ*nox*(C44A) ($P = 0.1147$, the log-rank test) (Figure 7E). Although the differences are not significant, it is quite obvious that the complementation strains of Δ*nox* with the inactive forms of NADH oxidase are attenuated compared with CΔ*nox* in the murine model of infection.

Overall, these results revealed that the enzymatic activity of NADH oxidase plays a key role in oxidative stress tolerance and a secondary role in virulence.

Discussion

Reactive oxygen species (ROS) generated by host phagocytes possess antimicrobial activity against a large number of pathogens.[36] The ability to survive oxidative stress is a key virulence-related trait in various bacteria.[37] Evidence is increasing that transcriptional regulation by Spx is important for low-GC Gram-positive bacteria to cope with oxidative stress.[8-11, 38, 39] We have recently shown that SpxA1 modulates oxidative stress tolerance and virulence in *S. suis*, and that a large number of genes are regulated by SpxA1.[11] In order to better understand the mechanism behind SpxA1 function, we targeted four genes (*0350*, *nox*, *tpx* and *copA*) that were expressed at significantly reduced levels in Δ*spxA1*,[11] to determine their specific roles in adaptation to oxidative stress and virulence potential.

In the preliminary study, the WT and mutant strains were cultured under different conditions to test the effect of each gene on oxidative stress tolerance. Genes *nox* and *tpx* are involved in oxidative stress tolerance,

while genes *0350* and *copA* have no major role in adaptation to the tested oxidative stresses. A murine infection model was adopted to evaluate the role of these genes in *S. suis* virulence. The data revealed that *nox* is required for infection. These results, coupled with previous study,[11] indicate that *nox* plays an important part in SpxA1 function. This is not surprising, as *S. suis* NADH oxidase shows considerable identity to its orthologs from various streptococcal species, which have been shown to be involved in oxidative stress response and virulence.[12-15, 29]

The Δ*nox* mutant exhibits reduced resistance to oxidative stress generated by environmental oxygen and hydrogen peroxide. This result is in agreement with the observed effects of *spxA1* deletion in *S. suis* and *nox2* deletion in Group B *Streptococcus*.[11, 13] In addition, *nox* has a role in tolerance to $ONOO^-$, which is indirectly generated by SIN-1 and represents a variant of highly reactive and bactericidal species.[33] Unlike the Group B *Streptococcus nox2* mutant that is hypersensitive to paraquat,[13] no paraquat tolerance phenotype was associated with *nox* deletion in *S. suis*. In *S. pneumoniae* and *Streptococcus mutans*, NADH oxidase acts to reduce diatomic oxygen to water through the oxidation of NADH to NAD^+, thus preventing formation of ROS.[12, 40] We therefore reasoned that NADH oxidase protects *S. suis* against oxidative stress via a similar mechanism.

NADH oxidase contributes to the pathogenesis of *S. pneumoniae*, Group B *Streptococcus* and *Streptococcus sanguinis*.[12, 13, 15, 29] In this study, we demonstrated that the virulence of *S. suis* 2 in a murine infection model was completely abolished by deletion of *nox*. The survival rates were significantly higher in mice infected with the Δ*nox* mutant than in those infected with the WT and CΔ*nox* strains. This observation could be explained by subsequent experiments, which showed that no bacterial cells could be recovered from the blood and organs of Δ*nox*-infected mice, and that a very low production of inflammatory cytokines was detected in Δ*nox*-infected mice. We also found that the ability of the Δ*nox* strain to grow in murine whole blood was significantly reduced. As NADH oxidase is involved in oxidative stress resistance, it is likely that Δ*nox* displays a defect in evasion of killing by phagocytes in the blood. The impaired growth of Δ*nox* in

blood might be partly responsible for its attenuated virulence. To further confirm the involvement of NADH oxidase in *S. suis* virulence, we performed an experimental infection of pigs. Animals inoculated with the WT and CΔ*nox* strains developed typical clinical symptoms and most of them died, while those inoculated with the Δ*nox* mutant displayed no clinical signs and all survived. In the brain, heart and lungs of pigs infected with the WT and CΔ*nox* strains, severe histopathological lesions were found. By contrast, the Δ*nox*-infected pigs exhibited no obvious pathological changes in these tissues. Together, these results indicated clearly that NADH oxidase is required for successful infection of *S. suis* 2. Despite the fact that NADH oxidase plays an important role in virulence of *S. suis* 2, it should be noted that some avirulent strains also harbor the *nox* gene. This observation may not be entirely surprising, as various virulence factors are present in *S. suis*,[41] and the infection process is complicated, depending on the cooperation of multiple factors.

To illustrate the involvement of the NADH oxidase activity in oxidative stress tolerance and virulence of *S. suis* 2, two inactive forms of NADH oxidase with point mutations of the His11 and Cys44 residues were generated. These two residues are the putative active sites of *S. suis* NADH oxidase, based on the structure of NADH oxidase of *S. pneumoniae* and *Streptococcus pyogenes*.[12, 42] Both inactive forms showed dramatically decreasing activity, suggesting that these two residues are the true active sites of *S. suis* NADH oxidase. Next, we constructed two complementation strains of Δ*nox* with the inactive NADH oxidase, and evaluated their abilities to resist to oxidative stress generated by environmental oxygen and hydrogen peroxide. Our results suggested clearly that the NADH oxidase activity has a key role in oxidative stress tolerance. It is also interesting to note that the complementation strains with the inactive NADH oxidase were more defective than the Δ*nox* mutant under oxidative stress conditions. We speculated that the presence of the inactive forms of NADH oxidase influenced the expression of other oxidative stress-related genes, such as *sodA* and *perR*.[26, 28, 43] Additionally, the survival rates of mice infected with the complementation strains with the inactive NADH oxidase were lower than those of Δ*nox*-infected mice, and higher than those of CΔ*nox*-infected mice, indicating that the NADH oxidase activity has a secondary role in virulence.

In conclusion, we have performed a study to examine the specific roles of four SpxA1-regulated genes in oxidative stress tolerance and virulence. We showed that the Δ*nox* strain is highly sensitive to oxidative stress agents. Using murine and pig infection models, we demonstrated that NADH oxidase is required for *S. suis* 2 infection. Moreover, the NADH oxidase activity has a key role in oxidative stress tolerance and a secondary role in virulence. Our results reveal that NADH oxidase plays an important part in SpxA1 function and provide a new insight into the pathogenesis of *S. suis* 2.

Materials and Methods

Bacterial strains, plasmids, primers and culture conditions

Bacterial strains and plasmids used in this study are listed in Supplementary Table 2. Primers are listed in Supplementary Table 3. *S. suis* strains were grown in Tryptic Soy Broth (TSB) or on Tryptic Soy Agar (TSA; Difco Laboratories, Detroit, MI, USA) with 10% (vol/vol) newborn bovine serum at 37ºC unless otherwise specified. *Escherichia coli* strains were cultured in Luria-Bertani (LB) broth or on LB agar at 37ºC. When required, antibiotics were added at the following concentrations: for *E. coli*, spectinomycin, 50 μg/mL; ampicillin, 50 μg/mL; kanamycin, 25 μg/mL; and for *S. suis*, spectinomycin, 100 μg/mL.

Construction of mutant strains and functional complementation of the *nox* deletion

In-frame deletion mutants of the genes *0350*, *nox*, *tpx* and *copA* were constructed in the SC19 background using the thermosensitive suicide plasmid pSET4s,[44] as previously described.[11] The complementation strain CΔ*nox* was generated using *E. coli-S. suis* shuttle vector pSET2,[45] as previously described.[46] The mutants (Δ*0350*, Δ*nox*, Δ*tpx* and Δ*copA*) and the complementation strain CΔ*nox* were verified by PCR (Supplementary Figure 4A), RT-PCR analysis (Supplementary Figure 4B) and direct DNA sequencing (data not shown).

Mutagenesis of NADH oxidase

The conserved residues His11 and Cys44 of NADH oxidase were changed separately to alanine using the Mut Express II Fast Mutagenesis Kit (Vazyme, Nanjing, China), according to the manufacturer's recommendations. A DNA fragment containing the *nox* gene and its predicted promoter was amplified from the *S. suis* 2 genome using primer pair Cnox1/Cnox2. The PCR product was purified and cloned into pMD18-T vector, to generate plasmid pMD18T-Cnox. Then, two pairs of primers containing the desired substitution amplified separately the entire sequence of pMD18T-Cnox. After digestion with *Dpn* I and homologous recombination, the products were transformed into *E. coli* Trans5α competent cells. The resulting plasmids were isolated and termed pMD18T-Cnox(H11A) and pMD18T-Cnox(C44A), respectively. Mutagenesis of the *nox* gene was verified by DNA sequencing.

Expression and Purification of recombinant proteins

The WT and mutant *nox* genes were amplified from the *S. suis* 2 genome, plasmids pMD18T-Cnox(H11A) and pMD18T-Cnox(C44A), respectively. The DNA fragments were digested with the *Bam*H I and *Hin*d III enzymes, and then cloned into pET-30a plasmid. After DNA sequencing, the resulting plasmids were transformed into *E. coli* BL21 (DE3) cells. When the cultures reached the exponential phase (OD_{600} = 0.6-0.8), 0.5 mM isopropyl b-D-1-thiogalactopyranoside (IPTG) was added to induce the expression of proteins. Then, the cells were grown for another 4 h at 28ºC before harvesting. The expressed proteins were purified by Ni-NTA affinity chromatography (GE Healthcare), according to the manufacturer's recommendations. The quality and concentrations of purified proteins were determined by SDS-PAGE and Qubit 2.0 fluorometer (Invitrogen), respectively. The proteins were stored at -80ºC until use.

Measurement of NADH oxidase activity

NADH oxidase activity was measured by monitoring the decrease in NADH absorbance (A_{340}) at 25ºC, as described previously.[29, 47] The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.2

mM EDTA, 0.2 mM NADH, and purified NADH oxidase. One unit of enzyme activity corresponds to the oxidation of 1 μmol of NADH per min at 25°C.

Complementation of the Δ*nox* mutant with the inactive forms of NADH oxidase

Primer pair Cnox1/Cnox2 amplified the DNA fragments containing the mutant *nox* gene and its predicted promoter from the plasmids pMD18T-Cnox(H11A) and pMD18T-Cnox(C44A), respectively. The DNA fragments were digested with the *Pst* I and *Bam*H I enzymes, and cloned into pSET2, to generate plasmids pSET2-*nox*(H11A) and pSET2-*nox*(C44A). After DNA sequencing, the plasmids were introduced into the Δ*nox* mutant by electroporation. The resulting complementation strains of Δ*nox* with the inactive NADH oxidase were selected with spectinomycin and designated CΔ*nox*(H11A) and CΔ*nox*(C44A), respectively.

Oxidative stress assays

To measure the susceptibility of *S. suis* strains (the WT, Δ*0350*, Δ*nox*, Δ*tpx* and Δ*copA*) toward oxidative stress, overnight cultures of each strain were diluted in fresh medium (TSB with 10% newborn bovine serum) and cultured at 37ºC under various conditions, including static conditions, vigorous shaking, and static conditions with 0.5 mM H_2O_2. Growth was monitored by measuring the optical density at 600 nm (OD_{600}) every hour.

To further investigate the role of NADH oxidase in oxidative stress tolerance, the WT, Δ*nox* and CΔ*nox* strains were subjected to a variety of oxidative stress challenges (vigorous shaking, 0.5 mM H_2O_2, 2 mM SIN-1, and 2 mM paraquat). Overnight cultures were diluted in fresh medium (TSB with 10% newborn bovine serum) adjusted to each specific condition, and growth was evaluated by measuring the OD_{600} every hour.

To determine whether NADH oxidase activity is involved in oxidative stress tolerance, the susceptibility of *S. suis* strains (the WT, Δ*nox*, CΔ*nox*, CΔ*nox*(H11A) and CΔ*nox*(C44A)) toward oxidative stress were tested as described above. Overnight cultures of each strain were diluted in fresh medium (TSB with 10% newborn

bovine serum) and cultured under various conditions, including static conditions, vigorous shaking, and static conditions with 0.5 mM H_2O_2. Growth was monitored by measuring the OD_{600} every hour.

Murine infection model

All animal studies were approved by the Laboratory Animal Monitoring Committee of Huazhong Agricultural University and conformed to the recommendations in the Guide for the Care and Use of Laboratory Animals of Hubei Province, China. Five-week-old female BALB/c mice (eight animals per group) were challenged i.p. with *S. suis* strains (the WT, Δ*0350*, Δ*nox*, Δ*tpx* and Δ*copA*) at a dose of approximately 1×10^8 CFU. The survival of mice was monitored twice daily for the first 2 days and daily for the next 5 days.

To further explore the role of NADH oxidase in virulence, five-week-old female BALB/c mice (10 animals per group) were inoculated i.p. with ~1.5×10^8 CFU of the WT, Δ*nox* and CΔ*nox* strains. Mice were monitored for 7 days for clinical signs and survival rates. For estimation of bacterial numbers in blood, brain, liver and spleen, mice were challenged i.p. with ~2×10^7 CFU of the WT, Δ*nox* and CΔ*nox* strains. At 6 and 12 h after challenge, five mice in each group were sacrificed, and bacterial numbers in blood and in homogenates of brain, liver and spleen were determined by plating.

To examine the involvement of NADH oxidase activity in virulence of *S. suis* 2, groups of eight BALB/c mice were inoculated i.p. with ~1.5×10^8 CFU of the WT, Δ*nox*, CΔ*nox*, CΔ*nox*(H11A) and CΔ*nox*(C44A) strains. The infected mice were monitored for clinical signs and survival time.

Measurement of inflammatory cytokines

To investigate the difference in cytokine release trigged by the WT, Δ*nox*, CΔ*nox* strains and the control P1/7, five-week-old female BALB/c mice were assigned randomly to four groups and each group was challenged i.p. with ~2×10^7 CFU of one of the indicated strains. At defined time points (3, 6, 9 and 12 h after infection), six mice per group were sacrificed and blood samples were collected by cardiac puncture. Serum samples were isolated by centrifugation and preserved at −80ºC until analysis. Levels of TNF-α and MCP-1 in serum

were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Neobioscience, Beijing, China), according to the manufacturer's recommendations.

Bactericidal assays

The bactericidal assays were performed as described elsewhere,[48] with some modifications. Heparinized whole blood was collected from BALB/c mice. The WT, Δ*nox* and CΔ*nox* strains were harvested at early stationary phase, washed twice with PBS, and diluted to 1×10^5 CFU/mL. Subsequently, bacterial suspensions (50 μL) were combined with fresh whole blood (450 μL), and the mixtures were incubated at 37ºC for 3 h with rotation. Aliquots were removed from the samples at hourly intervals and the number of viable bacteria was determined by plating. The growth factor was defined as the ratio of CFU in each sample after incubation over the CFU in the corresponding inoculum.

Experimental infection of pigs

A total of 20 high-health-status pigs (ages 4--5 weeks) which tested negative by ELISA for *S. suis* 2 were used. Pigs were randomly divided into four groups (five pigs per group). Animals in groups 1, 2 and 3 were inoculated by intravenous injection of ~1.3×10^6 CFU of the WT, Δ*nox* and CΔ*nox* strains, respectively. Group 4 was inoculated with PBS as a control. The infected pigs were monitored for clinical signs and survival time. Surviving animals were euthanized on day 7 post-infection. When the infected pigs died or pigs were humanely sacrificed, samples from the brain, heart and lungs were collected for pathological examination, as reported previously.[11]

Statistical analysis

Data were analyzed using GraphPad Prism 5 (San Diego, USA). Survival rates were analyzed by the log-rank test. Bacterial counts in the tissues of mice were analyzed by a repeated measures test with a Tukey post test. The Mann--Whitney test was used to analyze the production of cytokines in mice. Bacterial growth in blood

was analyzed using the two-tailed unpaired *t* test. The two-tailed paired *t* test was used for NADH oxidase activity analysis. A *P* value of < 0.05 was considered statistically significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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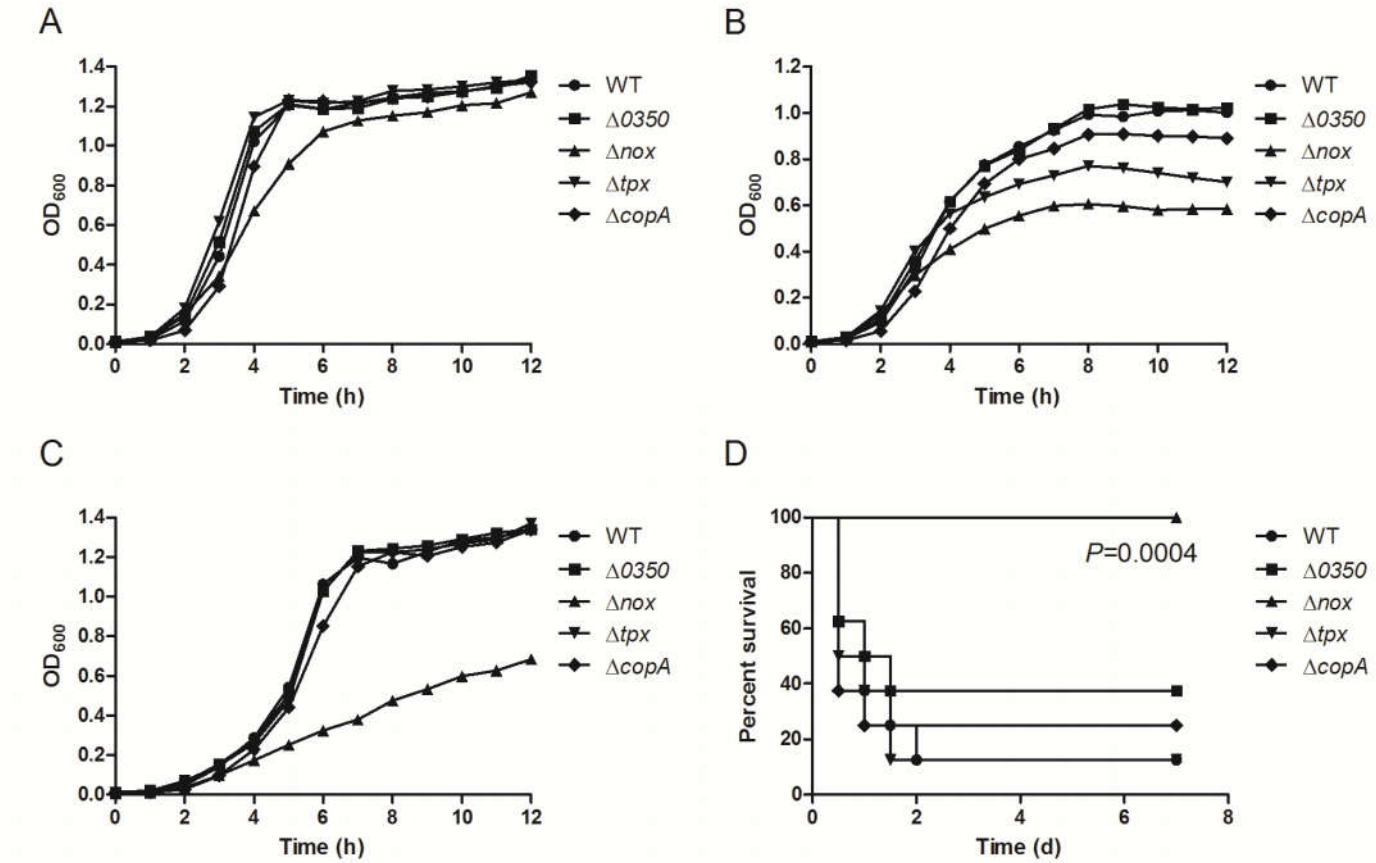

Figure 1. Preliminary research on the role of the genes *0350*, *nox*, *tpx* and *copA* in oxidative stress tolerance and virulence of *S. suis* 2. A, Growth curves of *S. suis* strains cultured under static conditions, i.e. low-oxygen conditions. B, Growth curves of *S. suis* strains cultured in a shaking incubator set to 200 rpm, i.e. high-oxygen conditions. C, Growth curves of *S. suis* strains cultured with 0.5 mM H_2O_2 under static conditions. Growth curves shown are representative of at least three independent experiments. D, Survival curves of mice infected with *S. suis* strains. Significant difference was observed between the WT and Δ*nox* group (P = 0.0004, the log-rank test).

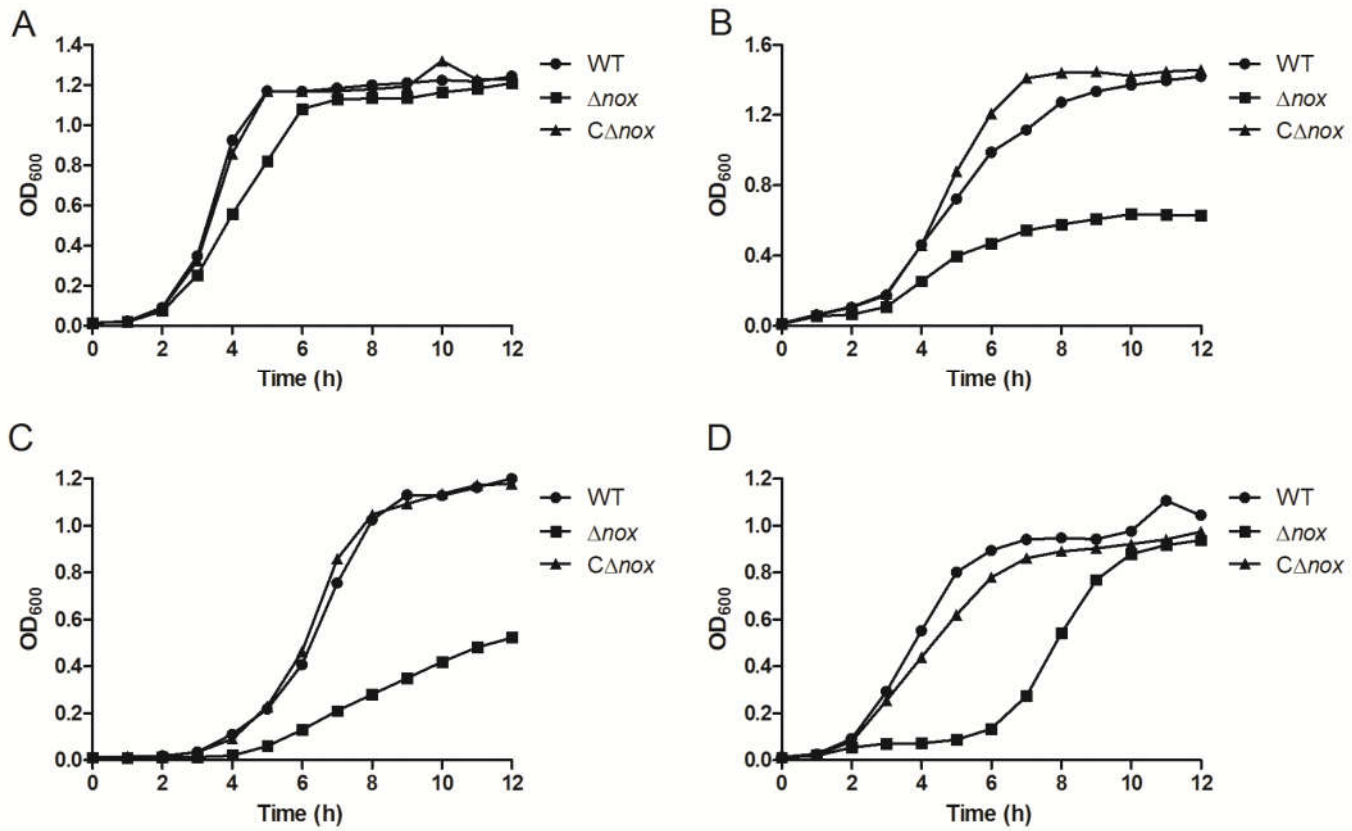

Figure 2. Growth characteristics of the WT, Δ*nox* and CΔ*nox* strains under various conditions. A, Growth under static conditions. B, Growth in a shaking incubator set to 200 rpm. C, Growth with 0.5 mM H_2O_2 under static conditions. D, Growth with 2 mM SIN-1 under static conditions. Growth curves shown are representative of at least three independent experiments.

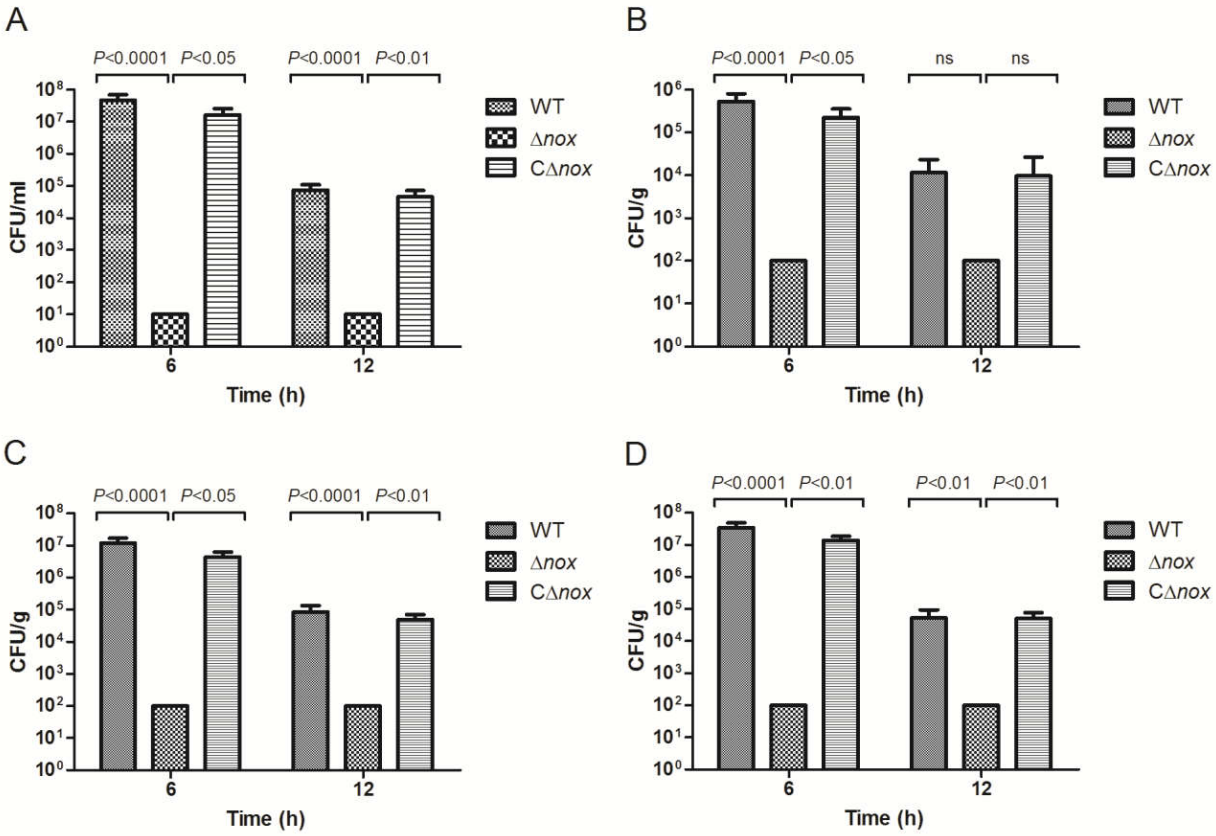

Figure 3. Colonization of various tissues of mice by the WT, Δ*nox* and CΔ*nox* strains. Mice were inoculated intraperitoneally with ~2×10^7 CFU of the WT, Δ*nox* and CΔ*nox* strains, respectively. Bacterial counts in the blood (A), brain (B), liver (C) and spleen (D) were examined at 6 h and 12 h post infection. The data shown are means with standard deviations for the results from two independent experiments. No bacterial cells could be recovered from mice in the Δ*nox* group, and the data shown are the limits of detection. Statistical analyses were performed by a repeated measures test with a Tukey post test. Significant differences were found at 6 h and 12 h between the Δ*nox* group and the WT group, and between the Δ*nox* group and the CΔ*nox* group for all tissues examined, with the exception of brain at 12 h.

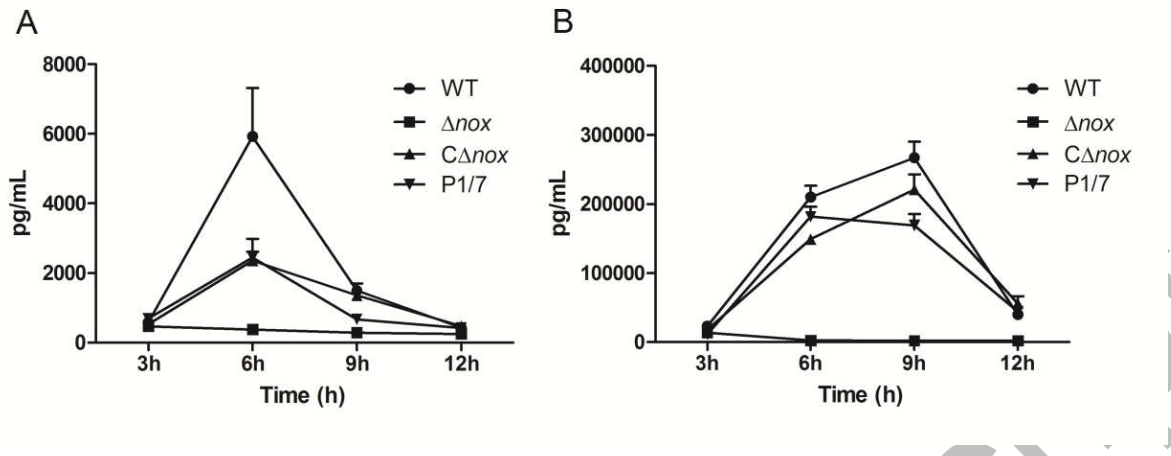

Figure 4. Time course of production of cytokines in mice infected with *S. suis* strains. A, Serum levels of TNF-α. Significant differences were found between Δ*nox* and the WT strain, and between Δ*nox* and CΔ*nox*, from 6 h to 12 h ($P < 0.01$). B, Serum levels of MCP-1. Significant differences were found between the WT and the mutant from 3 h to 12 h, and between the CΔ*nox* strain and the Δ*nox* mutant from 6 h to 12 h ($P < 0.01$). Data are expressed as means with standard error of the median from six mice for each strain at each time point. Statistical analyses were performed using the Mann--Whitney test.

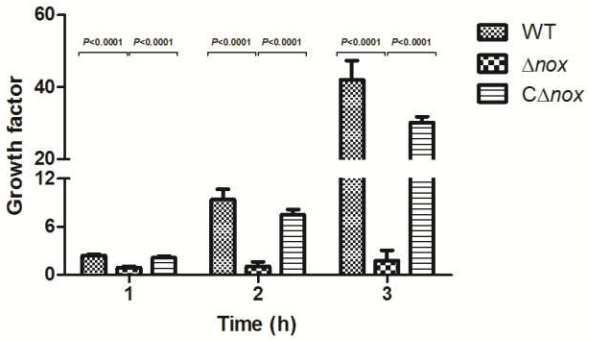

Figure 5. Growth factors of the WT, Δ*nox* and CΔ*nox* strains in mouse blood. The WT, Δ*nox* and CΔ*nox* strains were adjusted to 1×10^5 CFU/mL. Bacterial suspensions (50 μL) were combined with fresh whole blood (450 μL), and the mixtures were incubated at 37ºC for 3 h with end-to-end rotation. The growth factor was defined as the ratio of CFU in each sample after incubation over the CFU in the corresponding inoculum. The data shown are means with standard deviations for the results from three independent experiments carried out in duplicate. The two-tailed unpaired *t* test was used for statistical analysis. Significant differences were observed at 1, 2 and 3 h between the Δ*nox* group and the WT group, and between the Δ*nox* group and the CΔ*nox* group ($P < 0.0001$).

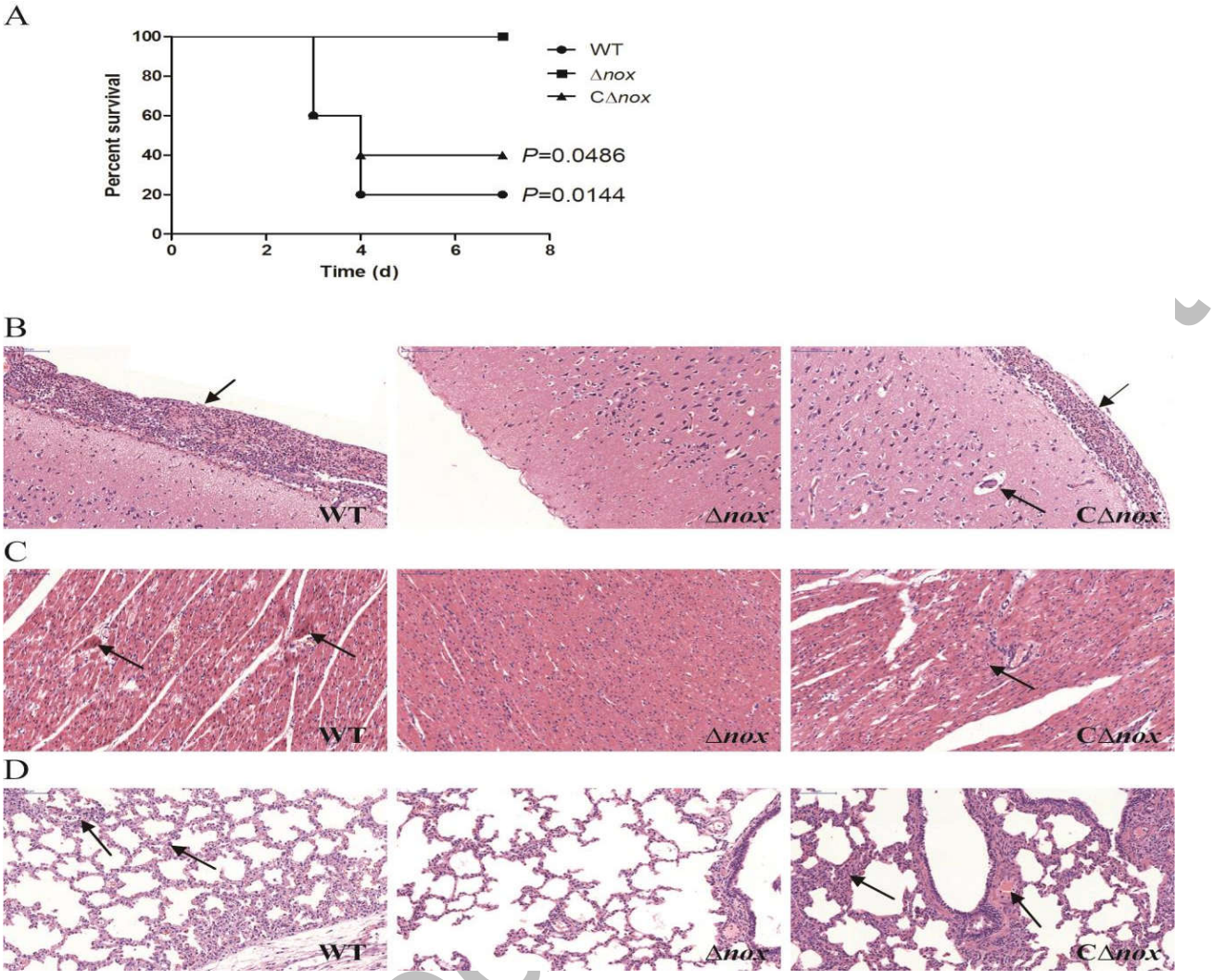

Figure 6. Role of NADH oxidase in *S. suis* 2 virulence in the pig infection model. A, Survival curves of pigs infected with *S. suis* strains. Animals inoculated with PBS are not shown for simplicity. $P = 0.0144$ for comparison of the Δ*nox* group with the WT group, and $P = 0.0486$ for comparison of the Δ*nox* group with the CΔ*nox* group (the log-rank test). B, Pathological examination of brain tissues of the infected pigs. C, Pathological examination of heart tissues of the infected pigs. D, Pathological examination of lungs tissues of the infected pigs. Arrowheads show the pathological changes. Representative images are shown for each group. Bars, 100 μm.

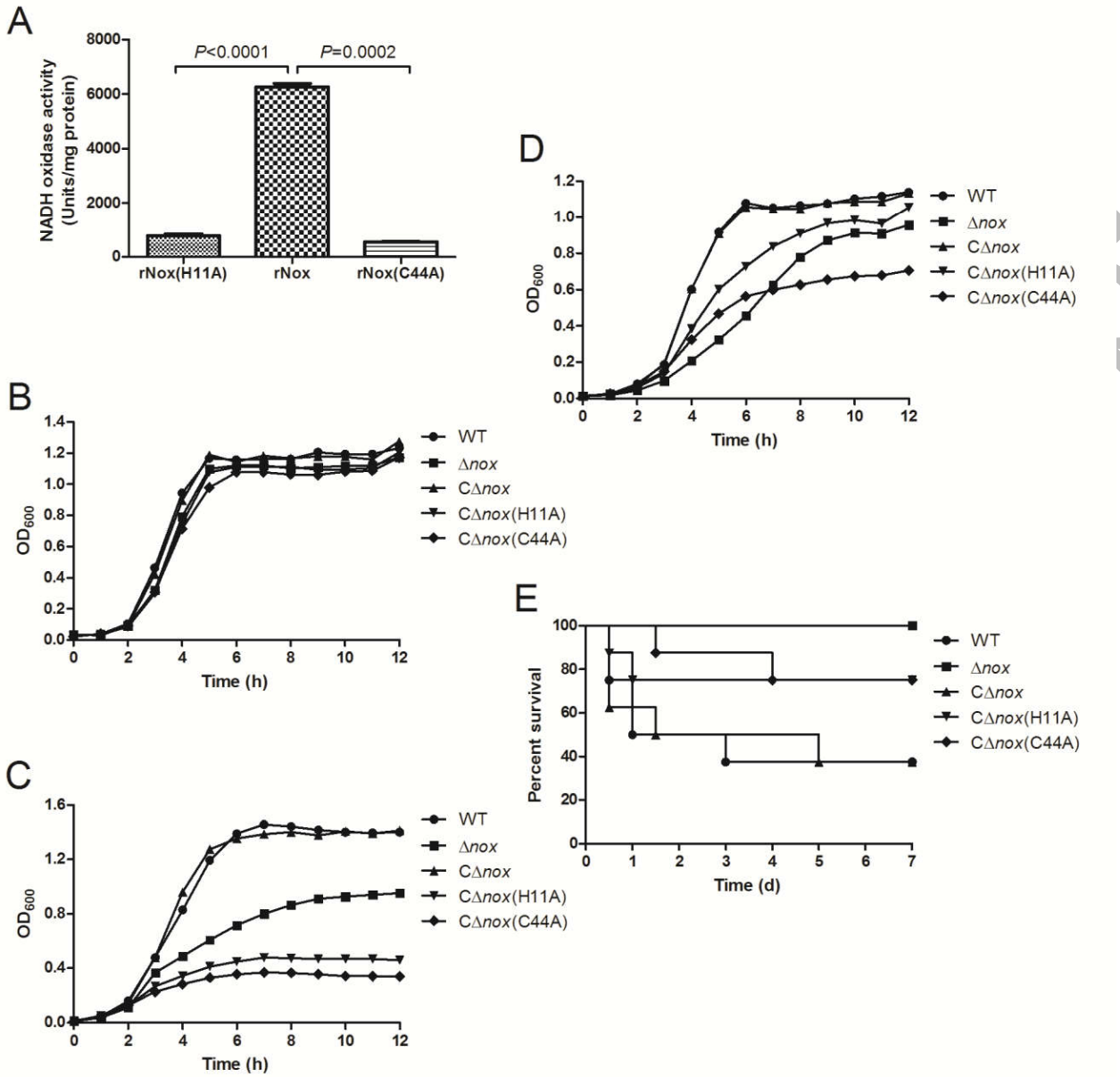

Figure 7. Role of the NADH oxidase activity in oxidative stress tolerance and virulence of *S. suis* 2. A, NADH oxidase activities of purified rNox, rNox(H11A) and rNox(C44A). The data shown are means with standard deviations of three independent experiments. Statistical analyses were performed using the two-tailed paired *t* test. B, Growth curves of *S. suis* strains cultured under static conditions. C, Growth curves of *S. suis* strains cultured in a shaking incubator set to 200 rpm. D, Growth curves of *S. suis* strains cultured with 0.5 mM H_2O_2 under static conditions. Growth curves shown are representative of at least three independent experiments. E, Survival curves of mice infected with *S. suis* strains. $P = 0.0082$ for comparison

of the CΔ*nox* group with the Δ*nox* group, $P = 0.1624$ for comparison of the CΔ*nox* group with the CΔ*nox*(H11A) group, and $P = 0.1147$ for comparison of the CΔ*nox* group with the CΔ*nox*(C44A) group (the log-rank test).